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Original Paper

Expression of the developmental Sonic hedgehog (Shh) signalling pathway is up-regulated in chronic lung fibrosis and the Shh receptor patched 1 is present in circulating T lymphocytes

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Abstract

During pulmonary development, Sonic hedgehog (Shh) and transforming growth factor β1 (TGF-β1) signalling both contribute to branching morphogenesis. In interstitial lung disease, the complex alveolar structure of the lung is disrupted and remodelled, which leads to fibrosis, loss of respiratory surface, morbidity, and mortality. It is well documented that TGF-β1 is involved in fibrosis. However, little is known about Shh signalling in damaged epithelia. This study examined whether or not components of the Shh signalling pathway, as well as TGF-β1, are expressed in human fibrotic lung disease (cryptogenic fibrosing alveolitis and bronchiectasis) and in murine experimental models of fibrotic and non-fibrotic chronic pulmonary inflammation. Using immunohistochemistry, it was observed that Shh, like TGF-β1, is up-regulated in epithelial cells at sites of fibrotic disease but not non-fibrotic inflammation. The Shh receptor patched was detected in infiltrating mononuclear cells and alveolar macrophages, as well as normal resting peripheral blood T lymphocytes. Neither Shh nor patched is expressed by hyperproliferative goblet cells in inflammatory epithelium. This study demonstrates that patched is present in human peripheral CD4 and CD8 lymphocytes at both protein and mRNA levels. Taken together, these results suggest that components of the highly conserved Shh signalling pathway may play a role in the remodelling of damaged pulmonary epithelium and that damaged epithelium and cells of the immune system may communicate via this pathway. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: interstitial lung disease; pulmonary fibrosis; inflammation; TGF-β1; developmental genes; Sonic hedgehog

Introduction

In the tissue remodelling response to pulmonary epithelial damage a balance is struck between closing the wound rapidly and repairing the respiratory surface. The remodelling involves interactions between several cell types including epithelial cells, fibroblasts, endothelial cells, and both resident and recruited cells of the immune system. The tissue attempts to replace damaged with new epithelial cells derived from a dividing population of alveolar type II cells. This process of regeneration is similar to events that occur during fetal lung branching and epithelialization [1–4].

Where the remodelling process fails to repair, fibrosis occurs with the formation of scar tissue [5–7]. In patients with interstitial lung disease (ILD), this remodelling is continuous and leads to interstitial fibrosis accompanied by a predominantly mononuclear lymphoid infiltrate in which both T and B lymphocytes are present [8–10]. In most cases of ILD, the nature of the damaging agent(s) is unknown [9] but an immunological origin has been presumed [10,11]. In cryptogenic fibrosing alveolitis (CFA), the commonest form of ILD, the disease is patchy and several stages of remodelling, repair, and scarring are seen simultaneously in sections of biopsy material [11–14]. All subsets of T lymphocytes are present. A relative increase in the number of CD8+ T cells has been correlated with a poorer prognosis [15]. Aggregates of B lymphocytes are also frequently observed at remodelling sites in the lungs of patients with CFA [14] and patients develop autoantibodies to pulmonary epithelial cell antigens [14,16–18]. Patients with CFA have

an average survival time of only 5 years post-diagnosis and there is currently no successful treatment that can halt the fibrotic process in these patients.

Patients may also develop pulmonary fibrosis secondary to chronic suppurative inflammatory processes where there is likely to be alveolar epithelial cell injury but no evidence to suggest a primary immunological pathogenesis. An example of such a condition is bronchiectasis, where larger conducting airways are primarily damaged due to inflammation and distal lung fibrosis may ensue due to chronic infection. In these cases, there may be extensive destructive fibrosis of the distal lung with an associated interstitial inflammatory infiltrate.

Many genes that are important in lung development are also up-regulated in fibrotic lung disease, such as TGF-β1 [19–23], tenascin [24,25], and integrins [26–28]. Some of these same genes are also involved in the regulation of immune system cell development and function including the TGF-β1 [29,30] and Sonic hedgehog (Shh) [31–34] signalling pathways. TGF-β1 is produced by a number of different cells in fibrotic lung, including epithelial cells and macrophages [23], while Shh has been reported to induce epithelial and mesenchymal hyperplasia [33,35]. Shh signals by interacting with its receptor patched (ptc), which activates the Gli family of transcription factors, which are also essential for lung development [36]. Binding of Shh to its receptor patched 1 (ptc 1) has been reported to inhibit a ptc 1-dependent G2/M checkpoint [37]. Thus, in remodelling lungs in chronic fibrosis, some of these molecules may allow cross-talk between damaged epithelial and immune system cells and may be involved in the recruitment and/or local survival of activated macrophages and T and B lymphocytes.

Since TGF-β1 and Shh both play a role in lung morphogenesis and epithelialization [30,31,33] and since TGF-β1 is known to be involved in pulmonary fibrosis [23,38], we were prompted to investigate whether or not the damage to epithelial cells in chronic fibrotic lung inflammation would induce expression of the Shh pathway. In this study, we investigated the expression of components of the Shh signalling pathway and TGF-β1 in different forms of chronic fibrotic lung disease. We examined biopsy material from patients with CFA or bronchiectasis. Two experimental models of murine lung inflammation were also studied: a murine model of ILD induced by intratracheal instillation of fluorescein isothiocyanate (FITC) [39] and a murine model of allergic airway inflammation induced by the group 1 allergen Der p1, derived from the house dust mite *Dermatophagoides pteronyssinus*.

Materials and methods

Biopsies

Ethical permission came from Lothian Universities NHS Trust Research Ethics Committee. Paraffin wax-embedded biopsy material from six patients with CFA and six with bronchiectasis came from the Department of Pathology archive.

RT-PCR

Human peripheral blood CD4+ and CD8+ T lymphocytes were enriched to more than 95% purity using MACS beads (Miltenyi Biotec Ltd, Bisley, Surrey, UK) according to the manufacturer's instructions. Total RNA was extracted and reverse-transcribed. The sequences for human patched were forward, 5′-CCATGTTCCAGTTAATGACTC-3′; reverse, 5′-ACATCATCCACACCAACA-3′. The reaction was optimized at 40 cycles with an annealing temperature of 58 °C. The product ran at the predicted size of 462 bp.

Animal models

All animal work was carried out with Home Office and local ethical committee approval.

Murine lung fibrosis

As previously published [39], female BALB/c mice (four per group) given an intratracheal instillation of 100 µg of fluorescein isothiocyanate in 50 µl of PBS or PBS alone were killed by phenobarbitone sodium (Sagatal, Merial Animal Health, UK) injection. After 7 days and 5 months, lungs were perfused with saline, distended with and fixed overnight in neutral formalin, and embedded paraffin wax.

Der p1-induced lung inflammation

Female C57BL/6 mice, 8 weeks of age, were immunized with affinity-purified Der p1 allergen from the house dust mite *Dermatophagoides pteronyssinus* intraperitoneally with 100 µl of saline containing Der p1 (10 µg) adsorbed to an equal volume of aluminium hydroxide adjuvant (Pierce, Rockford, IL, USA) on day 0 and day 17. On days 52 and 57, mice were given an intratracheal challenge of Der p1 (20 µg) in 50 µl of saline or of saline alone and killed 48 h later. For bronchoalveolar lavage (BAL), mice were exsanguinated and 500 µl of cold PBS was introduced via the trachea and withdrawn. BAL samples were centrifuged; the fluid was kept for cytokine measurement; and cytospins were made from the cells. The cytospins were stained with Diff-Quik (Gamidor Ltd, Abingdon, UK) for differential leukocyte population counting. For histology, lungs were distended with and fixed in formalin, and embedded in paraffin wax.

Cytokine ELISAs

Cytokines were measured in BAL fluid from Der p1-treated mice using Quantikine ELISA kits (R&D Systems Europe Ltd, Abingdon, UK) for interleukin

(IL)-4 (M4000) and IL-5 (M5000) following the manufacturer's protocol.

Histology

Three-micrometre sections were stained with haematoxylin and eosin for general morphology or Alcian blue/periodic acid Schiff for goblet cell hyperplasia.

Antibodies, sera, and immunohistochemistry

Microwave antigen retrieval (5 min in Antigen Retrieval Buffer, Vector Laboratories, Ltd) was used with all primary antibodies. Endogenous peroxidase was blocked by incubating the sections in aqueous 1% H_2O_2 for 5 min. Non-specific immunoglobulin staining was blocked by incubating sections with normal rabbit or goat serum for 30 min. Immunohistochemistry was performed on an automatic staining machine using peroxidase-labelled ABC with DAB visualization (Vector Laboratories, Ltd).

The following secondary antibodies and blocking sera were used: biotinylated-goat-anti-rabbit (1 : 400; DAKO UK Ltd, Ely, Cambridgeshire, UK), biotinylated-rabbit anti-goat IgG (1 : 400; DAKO) and -goat anti-chicken IgG (1 : 250; Vector Laboratories, Ltd, Peterborough, UK); normal goat, rabbit, mouse, and chicken serum (Scottish Antibody Production Unit, Carluke, UK); and normal human serum (Sigma-Aldrich Ltd, Fancy, Dorset, UK). Sections stained with secondary antibodies only to check peroxidase and non-specific immunoglobulin blocking were included in every run and were always negative. Primary antibodies to FITC (rabbit, 1 : 1000; DAKO), the N-terminus of Shh (goat, 1 : 40m dilution, 5 μg/ml, catalogue No sc1194), the C-terminus of ptc (goat, 1 : 40 dilution, 5 μg/ml, catalogue No sc6147) (Santa Cruz Biotechnology, Insight Biotechnology Ltd, Wembley, UK), and an antibody which neutralizes the activity of biologically active TGF-β1 but does not cross-react with TGF-β2 or -β3 (chicken, 1 : 50, catalogue No AF-101-NA, R&D Systems Europe, Ltd) recognized murine and human proteins. The sequence of the human peptide used to raise the anti-Shh antibody is identical to the murine sequence and the human peptide used to raise the anti-ptc antibody differs from the murine sequence by one amino acid (manufacturer's specifications); murine and human mature TGF-β1 differ by a single amino acid and are non-species-specific in their biological activity [40]. All antibodies were diluted in 20% normal human or mouse serum to block background on human or mouse tissue sections, respectively. Control sections, blocked in the same way, were stained with purified IgG from the same species as the primary antibodies at the same protein concentration and were always negative. Shh and ptc staining was blocked on control sections by pre-incubating the antibodies with the same peptide used to raise the antibodies (Santa Cruz Biotechnology) and as both antibodies were raised in goats and used at the same protein concentrations, their differing staining patterns acted as a further control for specificity.

Cytospins

Cells (100 μl of PBS containing 10^4 cells) were spun onto poly-L-lysine-coated microscope slides at 300 rpm for 3 min, using a Cytospin 3 (Shandon Scientific Ltd, Runcorn, UK). Following air-drying and methanol fixation, cytospins were stained with Diff-quick (Gamidor Ltd, Abingdon, UK) or used for immunohistochemistry.

Results

Pathology of FITC-induced lung fibrosis and Der p1-induced lung inflammation

As previously reported [39], FITC-induced fibrosis was associated with epithelial cell remodelling, the presence of type II alveolar cells, and interstitial infiltration of mononuclear cells at sites of immunohistochemically detected antigen deposition (Figures 1a–1c). No mucus-secreting goblet cells were detected in the airways (Figure 1d).

In contrast, Der p1-induced lung inflammation was characterized by marked goblet cell hyperplasia (Figure 1e) and granulocytic infiltration (Figure 1f). Further analysis of cells in BAL showed that there was a marked eosinophilia (Figure 2A) that was accompanied by a high level of IL-5 in BAL fluid (Figure 2B). Normal mice and control animals given PBS intratracheally had no mucin-positive cells in the airway. PBS-treated mice had fewer total cells present in BAL fluid ($176 \pm 30 \times 10^3$ per ml, $n = 7$) than Der p1-treated mice ($531 \pm 25 \times 10^3$ per ml, $n = 4$) and no eosinophils were detected (Figure 2A). Neither IL-4 nor IL-5 was detectable in the BAL fluid from PBS-treated mice.

The Shh pathway is up-regulated in murine lung fibrosis but not in Der p1-induced lung inflammation

Representative pictures from the fibrosis model are shown in Figures 3a–3i and from the Der p1 model in Figures 3j–3l. Six mice from each time point were examined and the same findings were seen in each, although the extent of fibrosis varied from animal to animal. Sections stained with normal goat serum or normal chicken serum under the same conditions were always negative (not shown).

In the fibrotic FITC model, immunohistochemical staining showed that ptc was present in bronchial and alveolar epithelial cells and on alveolar macrophages in both FITC-treated and PBS-treated mice. Ptc was also found on the predominantly mononuclear interstitial infiltrate seen at sites of immunohistochemically detected antigen deposition in the FITC-treated

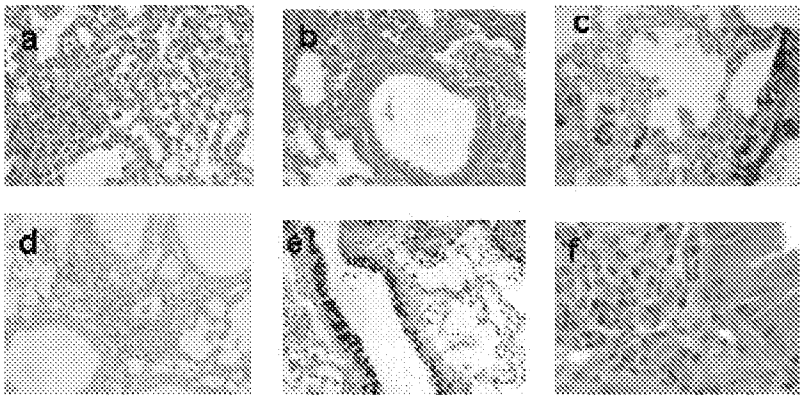

Figure 1. Murine lung inflammation. Haematoxylin and eosin-stained lung sections 7 days (a, original magnification ×400) and 5 months (b, original magnification ×400) after intratracheal instillation of FITC. Chronic, predominantly mononuclear inflammation and fibrosis are centred around sites of long-term FITC deposition shown by immunohistochemical detection of FITC 5 months after FITC instillation (c, original magnification ×400). There are no detectable mucin-containing goblet cells in the airways 5 months after FITC instillation (d, original magnification ×400). In contrast, airways in the lungs from Der p1-treated mice show goblet cell hyperplasia (e, original magnification ×400) and a predominantly granulocytic infiltrate (f, original magnification ×600)

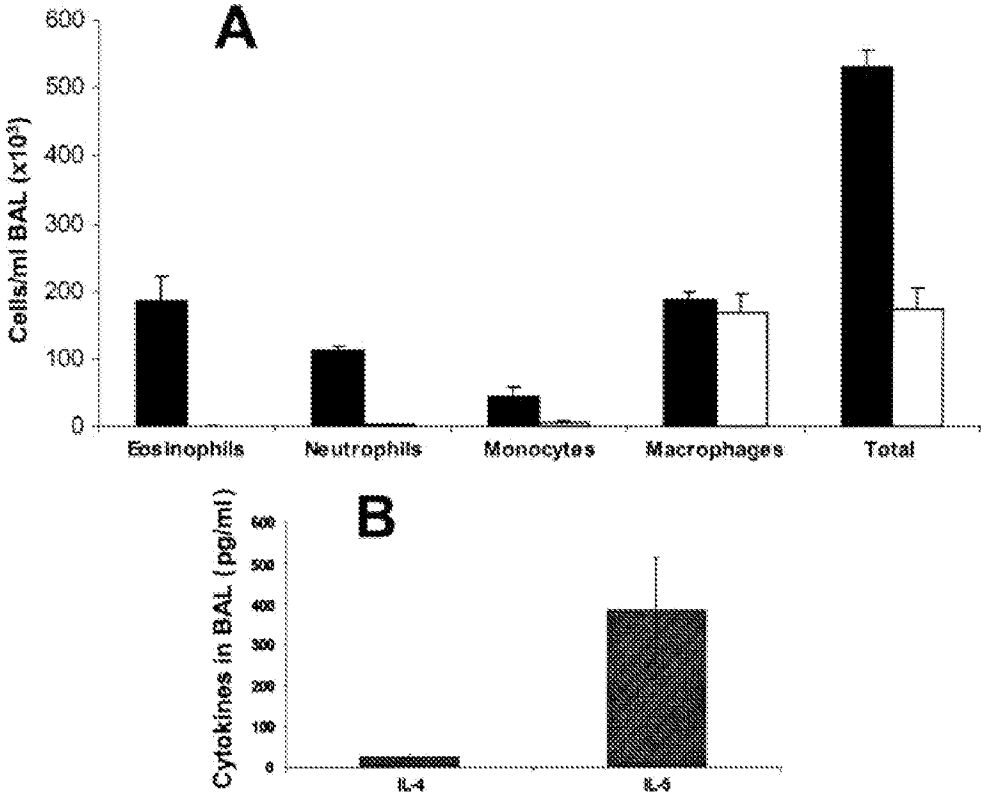

Figure 2. Mice immunized with Der p1/alum systemically and challenged by instillation of Der p1 into the airways exhibit increased numbers of total cells and eosinophils and elevated levels of IL-4 and IL-5 in BAL, compared with PBS-treated controls. (A) The number and type of cells present in the BAL fluid (×10^3): Der p1-treated mice, filled bars; PBS-treated mice, open bars. (B) IL-4 and IL-5 cytokine levels (pg/ml) in BAL from Der p1-treated mice. Data represent mean ± SEM of four mice

mice at both early (7 days) and late (5 months) time points (Figures 3a–3c). In contrast to the goat anti-ptc staining of PBS-treated lungs (Figure 3a), very weak or no goat anti-Shh staining was detected in epithelial cells of PBS-treated mice (Figure 3d), but up-regulation of Shh on bronchial and alveolar epithelial cells occurred within 7 days (Figure 3e) and was sustained in epithelial cells at sites of disease in lungs from mice given FITC intratracheally, although the infiltrating cells were predominantly negative (Figure 3f). TGF-β1 staining on alveolar macrophages and epithelial and infiltrating cells was marked in the

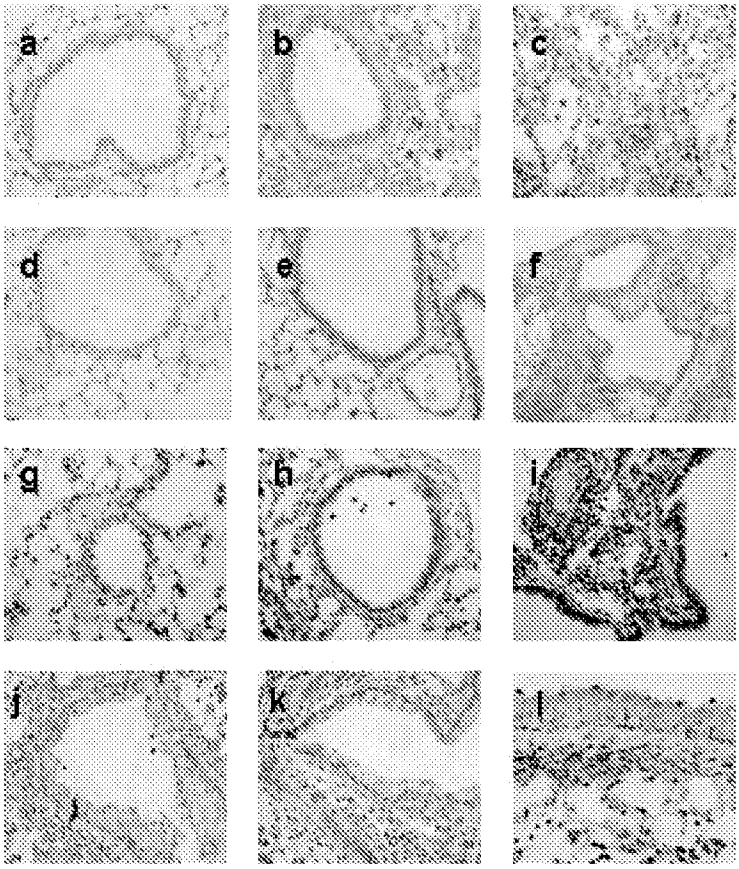

Figure 3. Representative ptc (a–c), Shh (d–f), and TGF-β1 (g–i) immunohistochemistry of murine lungs treated with PBS or FITC (original magnification ×400). Ptc staining is shown 7 days (a) after PBS and 7 days (b) and 5 months (c) after FITC treatment. Shh staining is shown 7days after PBS (d) and 7 days (e) and 5 months (f) after FITC treatment. TGF-β1 is shown 7 days after PBS (g) and 7 days (h) and 5 months (i) after FITC treatment. Representative ptc (j), shh (k), and TGF-β1 (l) immunohistochemistry of murine lungs after Der p1 treatment

FITC model at both early and late time points, but was only weakly present on epithelial cells alone in PBS-treated mice (Figures 3g–3i).

In contrast, lungs from mice given Der p1 had a predominantly granulocytic perivascular infiltrate and revealed minimal epithelial staining with either Shh or ptc, although ptc was present on mononuclear infiltrating cells (Figures 3j and 3k). The hyperplastic goblet cells in the Der p1 model were negative for both proteins. TGF-β1 staining was not detected in the goblet cells and was only weakly present in the epithelial cells (Figure 3l).

The Shh pathway is up-regulated in human interstitial lung fibrosis

In order to determine whether or not the findings in the mouse model were also seen in human disease, sections from lung biopsies from patients with CFA were examined. This condition causes patchy distal lung fibrosis accompanied by interstitial mononuclear cell infiltration. Normal goat serum showed no staining (Figure 4a). Figure 4 reveals that both ptc (Figure 4b) and Shh (Figure 4c) were expressed at diseased sites by cells with the classical morphological 'peg-like' appearance of hyperplastic type II epithelial cells. Shh was also expressed by ciliated metaplastic respiratory epithelial cells (Figure 4d). The distribution of ptc was fairly uniform, with cytoplasmic and membrane staining. Shh also showed cytoplasmic and membrane staining of epithelial cells but this was more focal in distribution at sites of active disease, particularly in the metaplastic respiratory epithelial cells, with positive and negative cells in juxtaposition as clearly illustrated in Figure 4d. In areas that appeared histologically normal, no Shh staining of epithelial cells was seen. As in the murine models, ptc was also expressed on alveolar macrophages and on infiltrating lymphoid cells (Figure 4e). Shh expression appeared restricted to abnormal areas of the lung tissue but ptc expression was observed at the alveolar surface of histologically normal areas of lung.

TGF-β1 staining was detected in both hyperplastic type II epithelial cells and metaplastic bronchial epithelial cells at sites of remodelling, in alveolar macrophages, and in infiltrating lymphoid cells (Figure 4f).

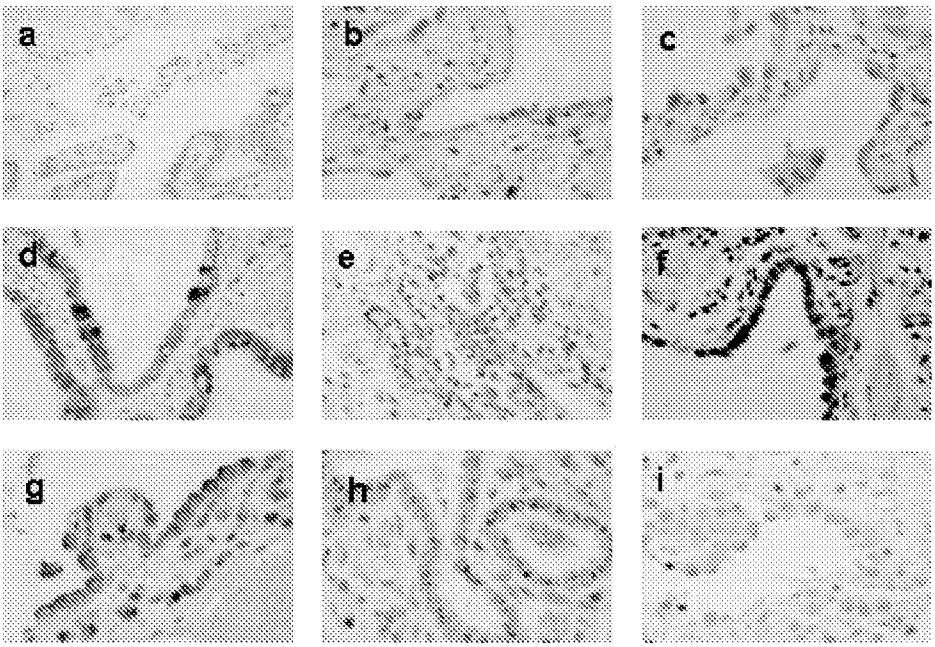

Figure 4. Sections from a lung biopsy taken from a patient diagnosed with CFA (a–f). No staining is seen with normal goat serum (a, original magnification ×400). Immunostaining for ptc on type II cells is shown in b (original magnification ×600). Shh staining of type II cells is shown at ×600 original magnification in c and Shh staining of metaplastic respiratory epithelial cells is shown at ×600 original magnification in d; ptc-positive cells are seen in the interstitial infiltrate (e) at ×600 original magnification. TGF-β1 immunostaining is seen in f at ×600 original magnification. Sections from lung tissue taken from a patient with bronchiectasis (g–i). Immunostaining for ptc (g), Shh (h), and TGF-β1 (i) (original magnification ×600)

The Shh hedgehog pathway is present in human bronchiectasis

In order to determine whether or not the expression of Shh was unique to CFA, biopsies from patients with bronchiectasis were also examined. This condition results in chronic suppurative inflammation of the lung with associated distal scarring beyond affected large airways. Shh was present in cells with the morphological features of hyperplastic type II epithelial cells at diseased sites in this condition, but revealed a distinct staining pattern with a uniform distribution and a granular 'perinuclear dot' staining pattern which was not seen in CFA (Figure 4g). Only two biopsies showed areas of 'normal' lung and in these areas no Shh staining was seen. Ptc had a similar diffuse pattern of staining to that seen in CFA (Figure 4h).

In contrast to the strong staining seen in CFA, TGF-β1 staining was only weakly present on epithelial cells in bronchiectasis (Figure 4i).

Ptc is present in resting human peripheral blood lymphocytes

Having found that Shh expression is up-regulated in damaged epithelial cells and that ptc is expressed on alveolar macrophages and infiltrating mononuclear cells, we were prompted to investigate if the expression of this signalling pathway is unique to immune system cells at an inflammatory site or a common feature of the peripheral immune system. To test this, we determined whether or not the receptor ptc was present on circulating cells in the peripheral blood. Figure 5a demonstrates membrane staining of the ptc receptor on CD4+ T cells isolated from peripheral blood and Figure 5b shows that both CD4 and CD8 T-cell subpopulations express *ptc 1* mRNA.

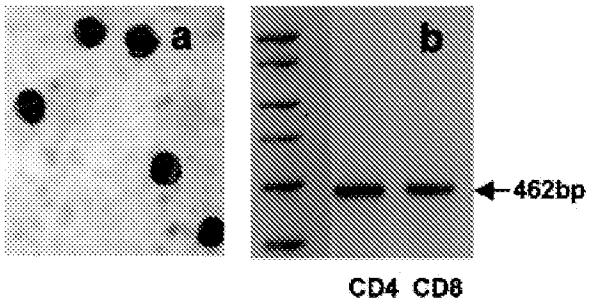

Figure 5. Ptc immunostaining on normal resting peripheral blood CD4+ T lymphocytes (a, original magnification ×1000). *Ptc* mRNA detected in both CD4+ and CD8+ peripheral blood lymphocytes by RT-PCR (b)

Discussion

In this study we report that alveolar macrophages and infiltrating lymphocytes express the Shh receptor ptc and that ptc is constitutively present at both mRNA and protein levels in circulating T lymphocytes. Furthermore, epithelial cells in the murine lung express ptc and its distribution on epithelial cells does not appear to alter in chronic lung inflammation. In

contrast, Shh expression by murine epithelial cells in our FITC model is up-regulated at sites of tissue remodelling and fibrosis. In human lung tissue, Shh protein is also markedly up-regulated on the surface and throughout the cytoplasm of remodelling epithelial cells in lungs of patients with CFA. Shh is also up-regulated in biopsies from patients with bronchiectasis, although with a different, granular perinuclear, pattern of staining. In the murine model of allergen-induced lung inflammation, only minimal staining for Shh is detected and the hyperplastic goblet cells characteristic of this model are negative. TGF-β1 protein is highly expressed in both CFA and the murine model of CFA at sites of disease, but is minimal in bronchiectasis and the murine model of allergic inflammation.

To our knowledge, this is the first description of expression of the Shh signalling pathway in chronic fibrotic lung disease. Shh is a member of a highly conserved family of morphogens originally described in *Drosophila* [41,42] and is critical in the normal development of the lungs [31]. Shh signals by interacting with its receptor ptc 1, which activates the Gli family of transcription factors, which are also essential for lung development [36]. Dysregulation of Shh signalling has been implicated in the development of basal cell carcinomas in mouse and man [43–46] and in cyst formation [47]. Shh has also recently been implicated in angiogenesis following trauma via the stimulation of VEGF secretion [48]. The role of TGF-β1 in regulating immune responses has been well documented (reviewed in refs 29, 49, and 50), whereas the role(s) of Shh is less well understood. Shh regulates the development of haematopoietic stem cells [32] and T lymphocytes in the thymus [34], and ptc has been detected in normal human bone marrow cells [51]. This indicates the importance of Shh signalling in the development of immune system cells, but it is not known whether or not Shh plays a role in the function of the mature immune system. The finding that mature T lymphocytes express ptc 1 suggests that this signalling pathway is functional in these cells and may allow immune cells to react to signals derived from damaged epithelial cells at sites of inflammation.

The finding that the Shh pathway, as well as TGF-β1, is up-regulated in both CFA and our murine model of pulmonary fibrosis implies that these pathways may recapitulate their developmental roles at sites of chronic damage. During lung development, these two pathways act antagonistically, with Shh promoting [31] and TGF-β1 inhibiting branching morphogenesis [19]. Since TGF-β1 promotes fibrosis in the lung [23,38,52,53], it may be that in a fibrotic context, Shh antagonizes this function and actively promotes epithelial repair. Alternatively, or possibly simultaneously, Shh may be involved in maintaining the inflammatory infiltrate at the sites of disease through the ptc receptors present on the surface of the infiltrating cells. The patchy pattern of staining with Shh in type II epithelial cells and particularly metaplastic respiratory epithelial cells in CFA, where positive and negative cells are in juxtaposition in areas, suggests that Shh expression is a very dynamic process in these cells.

A further observation is the very different pattern of Shh staining in patients with scarring of the distal lung secondary to bronchiectasis. The type II epithelial cells here showed prominent peri-nuclear staining for the protein, suggesting localization to the Golgi with only very occasional cells showing cytoplasmic or membranous staining. The development of fibrosis in this situation is a result of chronic suppurative infection rather than a primary immunological injury to the lung, as is believed to occur in CFA and our FITC model. This suggests that regulation of Shh expression in the presence of remodelling may be related to the type of injury applied. Further work aimed at understanding the mechanisms of action of Shh and its relationship to TGF-β1 xpression may improve our understanding of the complexities of chronic pulmonary fibrosis and the relative roles of epithelial, mesenchymal, and immune system cells in the disease process.

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